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Molecular cloning and tissue distribution of PHAS-I, an intracellular target for insulin and growth factors

(adipocyte/epidermal growth factor/protein kinase substrate/skeletal muscle)

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ABSTRACT **Although the actions of insulin and a number of growth factors that signal via protein-tyrosine kinase receptors are believed to involve increased phosphorylation of key intracellular proteins, relatively few of the downstream phosphoproteins have been identified. In this report we describe a cDNA encoding one of the most prominent insulin-stimulated phosphoproteins in rat adipocytes. The cDNA encodes a protein, designated PHAS-I, which has 117 amino acids and a M_r of 12,400. When translated *in vitro* and subjected to SDS/PAGE, PHAS-I migrates anomalously, having an apparent M_r of 21,000. The predicted amino acid composition is interesting in that approximately 45% of the PHAS-I protein is accounted for by only four amino acids—serine, threonine, proline, and glycine. The PHAS-I gene is expressed in a variety of tissues, although the highest levels of mRNA are present in fat and skeletal muscle, two of the most insulin-responsive tissues. The nucleotide and deduced amino acid sequences of PHAS-I differ from any that have been reported, and homology screening provided no clues concerning the function of the protein. However, in view of its tissue distribution and the fact that the protein is phosphorylated in response to insulin, we speculate that PHAS-I is important in insulin action.**

Investigations of the activation of glycogen synthase and pyruvate dehydrogenase by insulin led to the conclusion that the effects of insulin resulted from dephosphorylation of key intracellular enzymes (1, 2). The first direct evidence that insulin action was more complicated was obtained from experiments with ^{32}P-labeled adipocytes, where more proteins were found to be phosphorylated than dephosphorylated in response to the hormone (3, 4). It is now established that insulin action involves complex changes in the phosphorylation states of multiple intracellular proteins (5, 6). Several protein-serine/threonine kinases that are activated by insulin have been identified, and at least one important signal transduction pathway involves a phosphorylation cascade. This pathway, which is shared with several growth factors that signal via protein-tyrosine kinase receptors, involves the sequential activation of the serine/threonine kinases Raf-1, MEK, MAP kinase, and Rsk-II (7, 8). Identifying the downstream targets of this pathway and how the targets are regulated by phosphorylation will be essential in understanding how insulin regulates cell function.

The major insulin-stimulated phosphoproteins in adipocytes are phosphorylated primarily on serine and/or threonine residues (9, 10). One of the more prominent species undergoing increased phosphorylation in response to the hormone in ^{32}P-labeled fat cells is a heat- and acid-stable protein of apparent M_r 22,000, which we designate PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin). This protein is phosphorylated in multiple sites in response to insulin but is not phosphorylated in response to increased cAMP (11, 12). Moreover, in 3T3 L1 adipocytes, its phosphorylation has been shown to be increased by epidermal growth factor and platelet-derived growth factor, suggesting that it is involved not only in the action of insulin but also in the actions of several growth factors (13). In the experiments described here, we have cloned PHAS-I cDNA and demonstrated that the PHAS-I gene, which encodes a protein having a novel amino acid sequence,† is expressed in highest levels in skeletal muscle and fat cells, the major insulin-responsive cell types.

MATERIALS AND METHODS

Purification of PHAS-I. Adipocytes were isolated from adipose tissue of male Sprague–Dawley rats (180–200 g, 60 per preparation) (14). The cells (≈60 g) were washed twice and suspended (0.1 g of cells per ml) in low-P_i buffer (145 mM NaCl/5.4 mM KCl/1.4 mM $CaCl_2$/1.4 mM $MgSO_4$/0.1 mM sodium phosphate, pH 7.4) containing 3% (wt/vol) bovine serum albumin. Cells were incubated for 10 min at 37°C with insulin at 2.5 milliunits/ml, then rinsed twice in low-P_i buffer plus insulin, and homogenized in buffer (100 mM NaF/10 mM EDTA/2 mM EGTA/0.02% benzamidine/0.2 mM phenylmethylsulfonyl fluoride/50 mM Tris·HCl, pH 7.8; 1 mg/g of cells) in a glass homogenization tube with a Teflon pestle driven at 1000 rpm. To enable later detection of PHAS-I, a portion (10 ml) of the cells was labeled with ^{32}P (0.5 mCi/ml) for 2 hr (15), treated with insulin for 10 min, and homogenized. The ^{32}P-labeled and unlabeled homogenates were centrifuged at 10,000 × *g* for 30 min. The supernatants, excluding the fat cakes, were removed and centrifuged at 100,000 × *g* for 1 hr. The pellets were discarded and the extracts were incubated at 100°C for 5 min with vigorous mixing and then centrifuged at 100,000 × *g* for 1 hr to remove denatured protein. The heat-treated extracts were chilled to 0°C and trichloroacetic acid was added (1.5%). After 30 min, the samples were centrifuged at 100,000 × *g* to remove acid-insoluble material. The ^{32}P-labeled and unlabeled extracts were mixed and applied to a reverse-phase column (Vydac TP106, 4.6 × 250 mm) that had been equilibrated with 0.1% trifluoroacetic acid. Proteins were eluted at 1 ml/min with the following gradient of acetonitrile: 0% for 10 min, 0–60% in 60 min, 60–100% in 15 min, and 100% for 15 min. After SDS/PAGE, PHAS-I was readily identified by ^{32}P content and protein staining in fractions that were eluted at 43–45% acetonitrile. The protein was electroeluted from gel slices and concentrated by using an ISCO sample concentrator under conditions described previously (16).

Amino Acid Sequencing. PHAS-I (≈20 μg) from four preparations was subjected to SDS/PAGE and electrophoretically transferred to nitrocellulose (Schleicher & Schuell).

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U05014).

After Ponceau red staining, the PHAS-I band was excised and destained with 5% acetic acid (17). The slices were washed five times with water and then incubated at 37°C in 50 μl of 20 mM $CaCl_2$/1% (vol/vol) hydrogenated Triton X-100 (Calbiochem)/80 mM Tris Cl, pH 7.8. Three 5-μl aliquots of chymotrypsin (20 μg/ml; Worthington, 55 units/mg) were added at 8-hr intervals during the 24-hr incubation period. After the pH was adjusted to 2.1 with trifluoroacetic acid, the digest was applied to a microbore reverse-phase column (Applied Biosystems RP300) equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a linear gradient (0–90% in 100 min) of acetonitrile delivered at 100 μl/min. Absorbance at 212 nm was monitored, and peak fractions were manually collected for protein sequence analyses, which were conducted at the Washington University Protein Chemistry Laboratory. Peptides were sequenced with standard reagents and conditions for gas-phase chemistry on an automated protein sequencer (Applied Biosystems model 470A) equipped with an on-line UV detector to identify phenylthiohydantoin amino acid derivatives. The following peptides were sequenced: CT-1, XNVALGDGVQLPPGDY; CT-2, STTPGGTRIIY; CT-3, XNSPVAKPPPKDLLTIXGV; and CT-4, XSSPEDKRAGGEESQF.

Generation of a PHAS-I cDNA by PCR. Rapid amplification of cDNA 3′ ends (3′ RACE) was performed essentially as described (18, 19). In brief, cDNA template was generated by incubating a mixture (20 μl) containing 10 mM dNTPs, 10 μM $(dT)_{17}$ adapter (GIBCO/BRL), 2.5 μg of total RNA from isolated adipocytes, and 200 units of reverse transcriptase (GIBCO/BRL) for 30 min at 42°C. RNase H (2 units) was then added and the incubation was continued for 10 min. Oligonucleotides used as specific primers were constructed on the basis of frequency of codon usage in rat to correspond to best guesses for the cDNA encoding CT-1, CT-2, and CT-4. One round of PCR (25 cycles of 94°C for 45 sec, 50°C for 1.5 min, and 72°C for 2 min) was conducted with $(dT)_{17}$ adapter and each of the three specific primers. A sample of each of the first reaction mixtures was removed and used to prime a second round of PCR using the $(dT)_{17}$ adapter and each of the best-guess oligonucleotides as nested primers. When a 44-mer (5′-AACGTGGCCCTCGGCGACGGCGTGCAGCTGCCCCCCGGCGACTA-3′) encoding CT-1 was used in the first round, and a 44-mer (5′-AACAGCCCCGTGGCCAAGCCCCCCCCCAAGGACCTGCTGACCAT-3′) encoding CT-2 was used in the second, a 600-bp product was amplified. After subcloning into PCR II (Invitrogen), nucleotide sequencing revealed that the product contained a sequence that coded for CT-4.

DNA Library Screening and Nucleotide Sequencing. The PCR product was excised from the vector with *Eco*RI and purified by agarose gel electrophoresis. After radiolabeling with a random-primer kit (Promega), it was used to screen a poly(dT)-primed cDNA library prepared in λgt11 with poly(A)$^+$ RNA from isolated fat cells. A random-primed cDNA library (provided by John Merlie, Washington University) prepared in λZAP (Stratagene) from rat hindlimb muscle mRNA and a mixed poly(dT)-primed and random-primed cDNA library prepared in λgt11 from rat chest muscle mRNA (Clontech) were ultimately used to obtain the full-length PHAS-I cDNA. The libraries were screened by established procedures (20). In brief, phage were plated at 5–6 × 10^4 per 150-mm plate. Nitrocellulose filter replicas of 6–10 plates were prepared, and after denaturation and fixation of the DNA, the filters were incubated with the ^{32}P-labeled PCR product at 42°C for 24 hr. The filters were then washed for 1 hr at 65°C in 1× standard saline citrate (SSC) plus 0.1% SDS. Inserts from positive clones were subcloned into pBluescript SK(−) (Stratagene) for nucleotide sequencing. For clones appearing to be nearly full length, both strands were sequenced by using Sequenase (United States Biochemical). Automated sequence analyses (Applied Biosystems) were conducted to check the sequence presented in Fig. 2.

Computer-assisted searches for homologies between PHAS-I sequences and those of previously described nucleotide and amino acid sequences in GenBank release 77.0 and cumulative updates to the time the manuscript was submitted for review were performed with the BLAST and BLASTP network services at the National Center for Biotechnology Information. Screening for amino acid sequence patterns found in proteins of known function was conducted with the PROSITE program (IntelliGenetics).

Northern Blotting. Samples (20 μg) of total RNA isolated from tissues by extraction in guanidinium isothiocyanate and precipitation with ethanol (21) were subjected to electrophoresis in the presence of formaldehyde in 1% agarose gels. Smaller samples (2 μg) were subjected to electrophoresis in a duplicate gel, which was stained with ethidium bromide. The staining intensities of rRNA were measured to confirm that the samples contained equal amounts of total RNA. The RNA from the nonstained gel was transferred to a nitrocellulose sheet, which was incubated for 2 hr at 42°C with a solution containing 5× SSC, 5× Denhardt's solution, 50 μg of salmon sperm DNA per ml, and 50% (vol/vol) formamide. The sheet was then incubated with the same solution plus 10% (wt/vol) dextran sulfate and ^{32}P-labeled PHAS-I cDNA. After 18 hr the sheet was washed three times (15 min each) in 2× SSC/0.1% SDS at 23°C, once (10 min) in 0.25× SSC/0.1% SDS at 23°C, and once in 0.25× SSC/0.1% SDS at 62°C. Autoradiography was used to detect the hybridized probe.

***In Vitro* Translation and Immunoprecipitation.** pBluescript SK(−) containing PHAS-I inserts was linearized with either *Xba* I or *Stu* I and then used as template for generating PHAS-I mRNA with phage T7 RNA polymerase. A sample of the RNA formed was translated *in vitro* from [^{35}S]methionine and a mixture of nonradioactive amino acids by a rabbit reticulocyte lysate preparation (Promega). Antibodies to PHAS-I were generated by using a synthetic peptide (CSSPEDKRAGGEESQFE) having a sequence derived from the predicted carboxyl-terminal region of the protein. Rabbits were immunized with peptide coupled to keyhole limpet hemocyanin (22). Antibodies were affinity-purified before use by means of a column prepared with peptide coupled to SulfoLink resin (Pierce). For immunoprecipitation, antibodies were coupled to protein A-Sepharose beads (BRL; 0.5 mg of antibodies per ml of beads) by incubation at 23°C for 60 min in phosphate-buffered saline (PBS: 140 mM NaCl/5.4 mM KCl/10 mM sodium phosphate, pH 7.4). The beads were then washed three times with PBS. Samples (50 μl) of extract or *in vitro* translation product were incubated with beads (10 μl) for 30 min at 23°C with constant mixing. The beads were then washed three times in PBS/1% (vol/vol) Triton X-100 and once in PBS. Proteins were eluted by boiling the beads in SDS sample buffer and were subjected to SDS/PAGE (23).

RESULTS

Tissue Distribution of PHAS-I Gene Expression. A 600-bp product representing a partial PHAS-I cDNA was generated by rapid amplification of cDNA 3′ ends (3′ RACE) and used to screen a rat adipocyte cDNA library. Four clones were isolated that were identical over their entire lengths to regions of the PCR product; however, only one clone provided new sequence in the 5′ direction. This 712-bp cDNA did not represent the entire PHAS-I cDNA, as it lacked a start codon. To identify other tissues expressing the PHAS-I gene and to obtain an estimate of the size of the PHAS-I mRNA, a Northern blot was prepared and probed with the 712-bp cDNA. Total RNA samples from a variety of tissues were

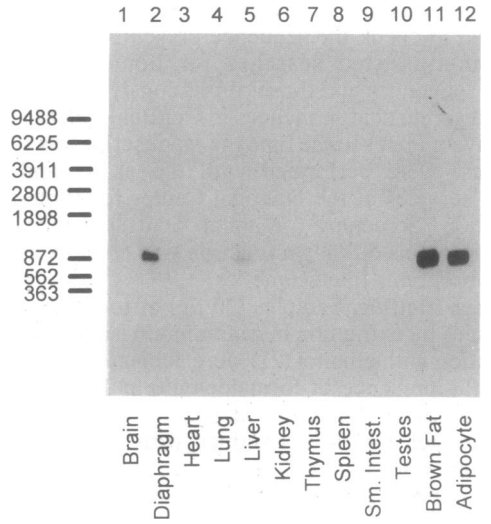

FIG. 1. Northern blot analysis of PHAS-I mRNA from various tissues. Samples of total RNA (20 μg) from isolated white adipocytes and the tissues indicated were subjected to denaturing electrophoresis in a 1% agarose gel. The RNA was then transferred to nitrocellulose and hybridized at 42°C with a ^{32}P-labeled probe prepared from a 712-bp cDNA cloned from an adipocyte library. The nitrocellulose sheet was washed at 62°C and exposed to x-ray film. A picture of the autoradiogram is presented. The positions and sizes (in nucleotides) of RNA standards are indicated. Sm. Intest., small intestine.

subjected to denaturing agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with the probe (Fig. 1). A species of ≈870 nt was detected in RNA samples from all of the tissues analyzed, with the exception of brain and testis, where little if any signal was detected. Expression was highest in adipose tissues, but relatively high levels were also detected in skeletal muscle.

PHAS-I cDNA. By screening a rat chest muscle cDNA library with a 400-bp fragment derived from the 5′ region of the largest adipocyte cDNA, one clone (from six that were characterized) was found that appeared to represent a full-length cDNA. The nucleotide sequence of this cDNA is presented in Fig. 2. A second cDNA, 1 bp shorter at the 5′ end and 202 bp shorter at the 3′ end, but having an otherwise identical sequence to the first, was one of 11 positive clones from a rat hindlimb cDNA library. The sequence of the 712-bp fat-cell cDNA was identical to a sequence found in the larger skeletal muscle clone.

The size of the PHAS-I cDNA, 844 bp, is very close to that predicted from the average size of the mRNA, when one considers the probability that the mRNA is more highly polyadenylylated than the cDNA. A consensus site (AAUAAA) (24) for binding of the cleavage and polyadenylylation specificity factor is located 40 bases upstream from the start of polyadenylylation (Fig. 2). A guanine residue is found in position −3 from the first ATG codon in the 5′ region. This methionine codon qualifies as a translation start site, as a purine in position −3 is the most important determinant for suitability of an AUG to serve as the site of translation initiation (25). The 5′ region of the cDNA preceding the first ATG has a very high G+C content (≈80%), suggesting that the predicted 5′ leader sequence is highly structured. The presumed ATG start site is found in an open reading frame extending to the stop codon at position 412. The predicted translation product is a 117-aa protein which contains all four chymotryptic peptides identified by amino acid sequencing. With the exception of Thr^{69}, amino acid sequences of the peptides deduced from the cDNA matched those determined by amino acid sequence analyses. Searches of GenBank with the deduced amino acid sequence of PHAS-I revealed only limited homology with other sequences. These seemed to represent chance homologies, as the sequences were all short proline-rich regions, which did not appear to fit a definable pattern.

The molecular weight of the PHAS-I protein encoded by the cDNA is 12,404, which is considerably less than that of the ^{32}P-labeled PHAS-I protein. Therefore, additional experiments were performed to confirm that the cDNA encoded the entire PHAS-I protein.

Confirmation of the PHAS-I Carboxyl Terminus. PHAS-I was purified from ^{32}P-labeled rat adipocytes as an insulin-stimulated phosphoprotein (Fig. 3). When subjected to SDS/PAGE, purified PHAS-I migrated slightly slower than soybean trypsin inhibitor, suggestive of a $M_r \approx 22{,}000$. Cleavage of the protein with endopeptidase Lys-C caused a decrease in mobility consistent with the loss of a M_r 1500 peptide from either the amino or the carboxyl terminus of the protein. Essentially all of the ^{32}P was recovered in the large protein fragment, demonstrating that the ^{32}P was on PHAS-I rather than on a contaminating protein of the same size. The intact protein yielded no sequence when subjected to Edman degradation, suggesting that its amino terminus was blocked. Attempts to sequence the large fragment were also unsuccessful, suggesting that the fragment retained the blocked amino terminus. When a sample of the digest was applied to the sequencer, a single sequence (XAGGEESQFEMDX)

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                                  5' GGGCCGAGGTGCCGCGGGGTTGCTGGAGGGTCGTGGGCGGCGTGCAGGAGAC    52

ATG TCG GCG GGC AGC AGT TGC AGC CAG ACT CCC AGC CGG GCT ATC CCC ACT CGC CGC GTA   112
MET Ser Ala Gly Ser Ser Cys Ser Gln Thr Pro Ser Arg Ala Ile Pro Thr Arg Arg Val    20

GCC CTC GGC GAC GGC GTG CAG CTC CCG CCC GGG GAC TAC AGC ACC ACC CCC GGC GGC ACG   172
Ala Leu Gly Asp Gly Val Gln Leu Pro Pro Gly Asp Tyr Ser Thr Thr Pro Gly Gly Thr    40

CTC TTC AGC ACC ACC CCG GGA GGA ACC AGA ATC ATC TAT GAC CGG AAA TTC CTG ATG GAG   232
Leu Phe Ser Thr Thr Pro Gly Gly Thr Arg Ile Ile Tyr Asp Arg Lys Phe Leu Met Glu    60

TGT CGG AAC TCG CCT GTG GCC AAA ACA CCC CCA AAG GAC CTG CCA ACC ATT CCA GGG GTC   292
Cys Arg Asn Ser Pro Val Ala Lys Thr Pro Pro Lys Asp Leu Pro Thr Ile Pro Gly Val    80

ACT AGC CCT ACC AGC GAT GAG CCT CCC ATG CAG GCC AGC CAG AGC CAT CTG CAC AGC AGC   352
Thr Ser Pro Thr Ser Asp Glu Pro Pro MET Gln Ala Ser Gln Ser His Leu His Ser Ser   100

CCG GAA GAT AAG CGG GCA GGT GGT GAA GAG TCA CAG TTT GAG ATG GAC ATT TAA GGGACCA   413
Pro Glu Asp Lys Arg Ala Gly Gly Glu Ser Gln Phe Glu Met Asp Ile ---              117

GCCATAGGACGCAGTGATGCTTCTGGGCCCCTGGGGCCCTTGGGAGGAGAGCCACAGCAGTCAGGCCTTGTACCCGGCA   492
GACACTGGGTGTGGATCGGCCACCCAGTCCTGCTCCTCACTCAGGGCACCTGCTCTGCCTTCCATTTTGTGAATACCAG   571
CACATACCTCCTTGTGCCTCTGTTGATACTGAGCTGCTACTCCAGGGTAATGACTCTCACCTACACCCTCCCTGCATCA   650
AGCGCCAGCGAGTGGACACAGAGGAGTCTGTCGGAATGATCTGGCAATTCTAGCCCCAACCTCTGGAGCACACCCACCT   729
TACCTTAGGTTGGGGTACCTGGGAAAGCCACCCTTTACTTCTTTCCCTGAGAGGAAATAAAAGCCACATTTACCCTAGG   808
CCCACAGCCGGGCCCTGTCTGAAAAAAAAAAAAAA 3'                                            843
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FIG. 2. Nucleotide sequence of a skeletal muscle cDNA clone and the amino acid sequence of the PHAS-I protein deduced from the cDNA are presented. Peptides identified by amino acid sequencing of chymotryptic fragments generated by digesting PHAS-I purified from adipocytes are denoted by bold type.

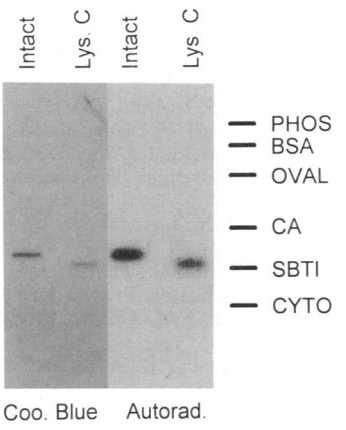

FIG. 3. Partial proteolysis of PHAS-I with endoproteinase Lys-C. A sample (≈5 μg) of PHAS-I purified from ^{32}P-labeled adipocytes was reduced with dithiothreitol and carboxyamidomethylated with iodoacetamide (26). The protein was incubated with endoproteinase Lys-C (0.2 μg) for 18 hr at 37°C in 50 μl of 0.1% SDS/0.1 mM $CaCl_2$/0.1 M NH_4HCO_3. Samples (≈0.2 μg) of the uncleaved ^{32}P-labeled PHAS-I and the Lys-C-treated protein were subjected to SDS/PAGE. The remainder of the digest was used for amino acid sequencing. Pictures of the Coomassie blue-stained gel and an autoradiogram are presented. Positions of molecular weight standards are to the right: PHOS, phosphorylase *b*; BSA, bovine serum albumin; OVAL, ovalbumin; CA, carbonic anhydrase; SBTI, soybean trypsin inhibitor; CYTO, cytochrome *c*.

was obtained which corresponded to the predicted carboxyl terminus of PHAS-I.

Immunoprecipitation and *in Vitro* Translation of PHAS-I. By use of antibodies to the carboxyl-terminal region of PHAS-I, a ^{32}P-labeled protein of apparent M_r 22,000 was immunoprecipitated from extracts of ^{32}P-labeled adipocytes (Fig. 4, lanes 1 and 2). None of the ^{32}P-labeled protein was recovered with nonimmune IgG. Incubation of cells with insulin increased the phosphorylation of the immunoprecipitated protein severalfold. The electrophoretic mobilities of the immunoprecipitated species were identical to those of the major heat- and acid-stable protein (which also migrates as a doublet) that is phosphorylated in response to insulin. These results indicate that PHAS-I antibody recognizes the appropriate ^{32}P-labeled protein.

To determine whether PHAS cDNA encoded a protein having the appropriate electrophoretic mobility, PHAS-I mRNA was synthesized *in vitro* by using T7 DNA-dependent RNA polymerase. The mRNA was then translated with [^{35}S]methionine in an *in vitro* translation reaction, and immunoprecipitations were performed with nonimmune IgG or PHAS-I antibody. An ^{35}S-labeled protein having an electrophoretic mobility in the presence of SDS slightly lower than that of the ^{32}P-labeled protein was recovered with PHAS-I antibody (Fig. 4, lanes 6–8), but not with nonimmune IgG (lanes 9–11). None of the ^{35}S-labeled protein was synthesized when PHAS-I mRNA was omitted from the translation reaction mixture (lane 5). These results indicate that PHAS-I migrates anomalously when subjected to SDS/PAGE, resulting in an apparent M_r that is 70% higher than its actual M_r of 12,400. Phosphorylation is known to retard the electrophoretic mobility of the adipocyte protein (11, 13), and the difference in the mobilities of the ^{35}S-labeled PHAS-I translated *in vitro* and the ^{32}P-labeled PHAS-I protein is most likely due to the presence of phosphate in the fat-cell protein. However, the effect of phosphorylation is too small to account for the large difference between the predicted and actual electrophoretic mobility of PHAS-I. Although the explanation for this difference is unclear, relatively large discrepancies between actual and apparent molecular

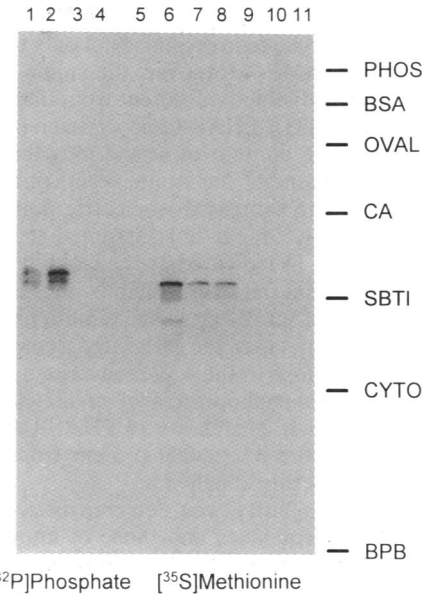

FIG. 4. Immunoprecipitation of PHAS-I from ^{32}P-labeled adipocytes and from *in vitro* translation reaction mixtures. Rat adipocytes were incubated in low-P_i medium containing $^{32}P_i$ for 2 hr and then were incubated without (lanes 1 and 3) and with (lanes 2 and 4) insulin at 2.5 milliunits/ml for 10 min before extracts were prepared. A plasmid containing the PHAS-I clone shown in Fig. 2 was linearized with *Stu* I (lanes 6 and 9). A plasmid containing a different PHAS-I clone isolated from a rat hindlimb muscle cDNA library was linearized with either *Xba* I (lanes 7 and 10) or *Stu* I (lanes 8 and 11). PHAS-I mRNA, transcribed from the cDNA by using T7 DNA-dependent RNA polymerase, was translated *in vitro* by using a rabbit reticulocyte lysate system and a mixture of amino acids containing [^{35}S]methionine. RNA was omitted from one translation reaction (lane 5). Immunoprecipitations were performed with nonimmune IgG (lanes 3, 4, and 9–11) or PHAS-I antibodies (lanes 1, 2, and 5–8) and samples were subjected to SDS/PAGE. Radioactivity in the dried gel was detected by using a PhosphorImager (Molecular Dynamics). Molecular weight markers are as in Fig. 3. The bromophenol blue (BPB) front is indicated.

weights have been observed for other proline-rich proteins (27).

DISCUSSION

We have cloned a cDNA encoding PHAS-I, one of the first insulin-stimulated phosphoproteins to be described (3, 13). Most of the information concerning PHAS-I relates to its properties as a target for phosphorylation in response to insulin and growth factors. The protein encoded by PHAS-I cDNA contains many potential sites of phosphorylation, and the 15 serine and 12 threonine residues which are present tend to be found in clusters. Thus, determining the exact residues that are phosphorylated in response to insulin is likely to be difficult. There is a protein kinase C consensus site (Thr^{17}) and four potential casein kinase II sites (Thr^{84}, Ser^{99}, Ser^{100}, and Ser^{111}) (28). Both of these kinases have been reported to be activated by insulin in adipocytes (29, 30). Interestingly, there are seven potential sites (Ser/Thr-Pro) (Thr^{10}, Thr^{36}, Thr^{44}, Ser^{64}, Thr^{69}, Ser^{82}, and Ser^{100}) for phosphorylation by MAP kinase (31), which is also activated in response to insulin and growth factors (7, 32). It will be interesting to determine whether PHAS-I is a substrate of these kinases.

No consensus sequence [RRX(S/T)] (28) for cAMP-dependent protein kinase is present in PHAS-I, consistent with the observation that the protein is not significantly phosphorylated in response to increased cAMP (11–13). This distinguishes PHAS-I from the heat- and acid-stable protein

of similar molecular weight described by Diggle and Denton (33) that is phosphorylated in response not only to insulin, but also to increased cAMP. Moreover, the amino acid composition of PHAS-I is distinctly different from that of the other heat-stable protein (33). PHAS-I has cysteine (two residues vs. none), twice the content of serine plus threonine, and about half the content of aspartate/asparagine plus glutamate/glutamine. The estimated isoelectric point of the unmodified PHAS-I is 5.46. If it is assumed that the amino terminus is blocked, as indicated by the inability to sequence the intact protein, and that the protein is phosphorylated on two sites, to account for the presence of both phosphoserine and phosphothreonine, then the calculated isoelectric point is 4.95. This is quite close to the 4.92 measured by Blackshear *et al.* (12) for the phosphoprotein of M_r 22,000 which we believe to be PHAS-I. The ability of PHAS-I antibodies to immunoprecipitate the M_r 22,000 protein from ^{32}P-labeled adipocytes supports this conclusion.

No significant similarity was detected between PHAS-I cDNA or protein sequences and those of known proteins. Thus, homology screening did not provide a clue concerning the function of PHAS-I. However, the deduced amino acid sequence of PHAS-I is interesting in at least two respects. Approximately 45% of the PHAS-I protein is accounted for by only four amino acids—serine, threonine, proline, and glycine. Between residues 33 and 49 there is a repeat of the following sequence: (Phe/Tyr)-Ser-Thr-Thr-Pro-Gly-Gly-Thr. The repeat is contained in one (Val^{20}–Thr^{49}) of two "PEST" regions in PHAS-I, although the second such region (Asp^{73}–Ser^{95}) appears to conform better to the PEST consensus sequence (34). Whether the repeated motif is of functional significance is not known, although the presence of PEST motifs indicates that PHAS-I may be rapidly degraded in cells (34). The very high G+C content of the mRNA leader sequence has potential implications for the expression of the protein, as the translation efficiency of such messages is generally poor (25). The list of vertebrate mRNAs with G+C-rich leader sequences includes many receptor proteins, oncogenes, and signal transduction components (25). In view of the selective expression of the PHAS-I gene in skeletal muscle and fat cells, which are the insulin-responsive cell types, and the regulation of PHAS-I phosphorylation by insulin, we speculate that PHAS-I is a component of the insulin signal transduction pathway.

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